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REMARKS

Claims 65, 75 and 76 are pending in this application, claims 75 and 76 having been added by applicants and claims 51 to 64 and 66 to 74 having been withdrawn by the Examiner. Claim 65 has been amended to recite a method of treating rheumatoid arthritis in a patient, which includes administering a therapeutically effective amount of a reagent that inhibits mitogen-activated protein kinase kinase 3 (MKK3) activity. Support for this amendment can be found throughout the specification, e.g., at page 10, lines 1 to 27. Support for new claims 75 and 76 can be found, for example, at page 47, lines 27 to 29.

As instructed by the Examiner at items 3, 4, 5, and 8 of the Office Action, applicants have amended the application title, the paragraph entitled "Cross-Reference to Related Application," several paragraphs of the specification that refer to trademarks, and claim 65 (to spell out the abbreviation "MKK").

The amendments discussed above add no new matter to the present application.

35 U.S.C. 112, paragraph 1

Claim 65 has been rejected for an alleged lack of enablement. As mentioned above, applicants have amended claim 65 to recite a method of treating rheumatoid arthritis in a patient and to recite that the method includes administering to the patient a therapeutically effective amount of an antibody that inhibits MKK3 activity. Applicants respectfully traverse this rejection with respect to amended claim 65 for the reasons discussed below.

To support this rejection, the Office Action states (at page 4):

> In view of the lack of predictability of the art to which the invention pertains the lack of established clinical protocols for effective therapies for antibodies that inhibit kinases and, in turn, inhibit inflammatory conditions, including rheumatoid arthritis, undue experimentation would be required to practice the claimed methods with a reasonable expectations of success, absent a specific and detailed description in applicant's evidence which is reasonably predictive that the claimed methods are effective for inhibiting MKK3-mediated disorders broadly encompassed by the claimed invention.

In further support of the rejection, the Office Action cites two publications (Bokemeyer et al. and Wolf et al.), which discuss the role of extracellular signal-regulated kinase (ERK) in certain diseases.

As an initial matter, applicants respectfully submit that Bokemeyer and Wolf are not probative on the issue of whether applicants' claimed method is enabled. Neither Bokemeyer nor Wolf discuss the role of MKKs in disease states in any particular detail, nor do they discuss whether inhibitors of MKKs would be useful for treating certain diseases. In fact, neither of these publications appear to discuss the role of MKK3 in rheumatoid arthritis at all. Rather, Bokemeyer and Wolf discuss the role of extracellular signal-regulated kinases (ERKs) in diseases such as mesangioproliferative glomerulonephritis, human malignancies, and cardiac hypertrophy, among many others, and the potential for using ERK signaling cascade inhibitors to treat such diseases. Accordingly, applicants submit that these two references do not support the Office Action's assertion that applicants' claimed method of treating disorders is not enabled by the specification as filed.

On the contrary, applicants' amended claim 65 is enabled by the teachings provided in the specification and by the knowledge of those skilled in the art. For example, the specification indicates and provides examples demonstrating that MKK3 phosphorylates (activates) p38 in cells under a variety of conditions (see, e.g., Examples 5 and 6 and the specification at page 16, line 8 to page 17, line 2). At the time the application was filed, p38 was known to increase expression of inflammatory cytokines (see Raingeaud et al., J. Biol Chem. Vol. 270(13):7420-7426 (1995); cited as item "AS" in applicants information disclosure statement filed August 16, 2001, a copy of which is attached hereto as Exhibit A). Thus, any skilled practitioner would have appreciated that an inhibitor of MKK3 activity could be used to reduce expression of inflammatory cytokines and, therefore, inflammatory disorders in general. Indeed, the specification emphasizes this point, teaching (at page 10, lines 1 to 11, page 46, lines 16 to 20, and page 47, lines 8 to 11) that inflammatory disorders, such as rheumatoid arthritis (RA), can be treated by administering MKK inhibitors described in the specification, which would act to inhibit secretion of inflammatory cytokines including TNF and IL-1. Applicants submit that

based on the information provided in the specification alone, skilled practitioners would have appreciated that an inhibitor of MKK3, such as an antibody, could be used to treat RA.

Moreover, applicants' assertion that MKKs are involved in RA was corroborated recently in the publication Chabaud-Riou et al., Am. J. Path Vol 164(1):177-184 (2004) (a copy of which is attached hereto as Exhibit B), which describes studies demonstrating that MKK3 and MKK6 are important regulators of p38 in fibroblast-like synoviocytes and are activated in RA synovium. This finding leads Chabaud-Riou to state that MKK3 and MKK6 are potential therapeutic targets for treating RA (see Chabaud-Riou at page 178, column 1).

In addition, the specification provides detailed teachings about production of anti-MKK antibodies (see, e.g., the specification at page 6, line 26, to page 7, line 17), production of preparations of antibodies for administration to patients (see, e.g., page 48, lines 12 to 33), and dosage (see, e.g., page 46, lines 16 to 32).

Accordingly, applicants submit that there can be no issue about enablement of the present claim. For one skilled in the art, the application provides all the information necessary for the practitioner to appreciate the role of MKK3 in rheumatoid arthritis, to formulate anti-MKK3 antibodies as pharmaceutical compositions, and then use antibodies to treat rheumatoid arthritis, without undue experimentation. Applicants respectfully submit that this rejection should be reconsidered and withdrawn.

35 U.S.C. 112, paragraph 2

Claim 65 has been rejected as allegedly indefinite for recitation of the phrases "MKK-mediated disorders" and "modulates MKK activity." Applicants respectfully disagree and submit that the metes and bounds of original claim 65 are clear. However, for reasons unrelated to the present rejection, applicants have amended claim 65 to delete both of these phrases. Applicants submit that this amendment obviates the present rejection and request that it be withdrawn.

CONCLUSION

Applicants submit that all claims are in condition for allowance, which action is requested. Enclosed is a $950 check for the Petition for Extension of Time fee. Please apply any other charges or credits to deposit account 06-1050, referencing Attorney Docket No. 10363-003004.

Respectfully submitted,

Date: 7/27/04

REG. NO. 54,112

J. Peter Fasse
Reg. No. 32,983

Fish & Richardson P.C.
225 Franklin Street
Boston, MA 02110-2804
Telephone: (617) 542-5070
Facsimile: (617) 542-8906

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Pro-inflammatory Cytokines and Environmental Stress Cause p38 Mitogen-activated Protein Kinase Activation by Dual Phosphorylation on Tyrosine and Threonine*

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Joël Raingeaud‡, Shashi Gupta‡, Jeffrey S. Rogers‡, Martin Dickens‡, Jiahuai Han§, Richard J. Ulevitch§, and Roger J. Davis‡¶‖

From the ‡Program in Molecular Medicine, Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01605, the ¶Howard Hughes Medical Institute, Worcester, Massachusetts 01605, and the §Department of Immunology, The Scripps Research Institute, La Jolla, California 92037

Protein kinases activated by dual phosphorylation on Tyr and Thr (MAP kinases) can be grouped into two major classes: ERK and JNK. The ERK group regulates multiple targets in response to growth factors via a Ras-dependent mechanism. In contrast, JNK activates the transcription factor c-Jun in response to pro-inflammatory cytokines and exposure of cells to several forms of environmental stress. Recently, a novel mammalian protein kinase (p38) that shares sequence similarity with mitogen-activated protein (MAP) kinases was identified. Here, we demonstrate that p38, like JNK, is activated by treatment of cells with pro-inflammatory cytokines and environmental stress. The mechanism of p38 activation is mediated by dual phosphorylation on Thr-180 and Tyr-182. Immunofluorescence microscopy demonstrated that p38 MAP kinase is present in both the nucleus and cytoplasm of activated cells. Together, these data establish that p38 is a member of the mammalian MAP kinase group.

Several MAP[1] kinase signal transduction pathways have been molecularly characterized (1). At least four genetically distinct signaling pathways have been defined in the yeast *Saccharomyces cerevisiae* (2). One pathway leads to activation of the FUS3 and KSS1 MAP kinases and is required for the response to mating pheromone (3). A second MAP kinase pathway (MPK1) functions during cell wall biosynthesis (4, 5). A third genetically defined MAP kinase pathway (HOG1) is involved in osmoregulation (6). The fourth MAP kinase pathway (SMK1) is required for the control of sporulation (7). Significantly, these MAP kinase pathways appear to function independently because mutations that disrupt one pathway do not alter signal transduction mediated by the other pathways (2). This independent function may arise from the substrate specificity of the MAP kinase cascades. In addition, it has been established that there is an important role for tethering proteins (*e.g.* STE5) that bind multiple components of the MAP kinase cascade to create a functional signal transduction module (8, 9).

Although detailed information is available for yeast, the organization of MAP kinase pathways in mammals is more poorly understood. The ERK group of MAP kinases is activated by growth factors via a Ras-dependent signal transduction pathway (10). In contrast, the JNK group of MAP kinases (also designated SAPK) is activated by pro-inflammatory cytokines and environmental stress (11–17). JNK activation is also observed during co-stimulation of T lymphocytes (18). Importantly, the signal transduction pathways that lead to ERK and JNK activation are biochemically and functionally distinct (11).

Recently, a novel mammalian MAP kinase (p38) was identified by Han *et al.* (19). This MAP kinase isoform has been implicated in the mechanism of activation of MAPKAP kinase-2 (20, 21) and the expression of pro-inflammatory cytokines (22). Homologs of p38 MAP kinase (CSBP1 and CSBP2) have been identified in human tissues (22). A p38 MAP kinase homolog (MPK2) has also been identified in *Xenopus laevis* (20). The purpose of this study was to examine the mechanism of p38 activation and to establish the relationship of the p38 MAP kinase pathway to the ERK and JNK signal transduction pathways.

EXPERIMENTAL PROCEDURES

Materials—Tumor necrosis factor α and interleukin-1α were from Genzyme Corp. Lipolysaccharide (LPS) was isolated from lyophilized *Salmonella minesota* Re595 bacteria as described (23). Phorbol myristate acetate was from Sigma. EGF was purified from mouse salivary glands (24). The monoclonal antibodies M2 and PY20 were obtained from IBI-Kodak and ICN, respectively. [^{32}P]ATP was prepared using a Gammaprep A kit (Promega Biotech) and [^{32}P]phosphate (DuPont NEN). Recombinant ATF2 proteins have been described (25). GST-IκB was provided by Dr. D. Baltimore (Massachusetts Institute of Technology). GST-c-Myc (26), GST-EGF-R (residues 647–688) (27), and GST-c-Jun (11) fusion proteins have been described. GST-p38 MAP kinase was prepared using the expression vector pGEX and a polymerase chain reaction fragment containing the coding region of the p38 MAP kinase cDNA. The GST fusion proteins were purified by affinity chromatography using gluthathione-agarose (28). Polyclonal antibodies that recognize JNK and p38 MAP kinase were raised in rabbits using GST-p38 and GST-JNK1 as antigens.

The plasmid pCMV-Flag-JNK1 (11) and the expression vectors for human MKP-1 (CL100) and PAC-1 (29) have been described. The plasmid pCMV-Flag-p38 MAP kinase was prepared using the expression vector pCMV5 (30) and the p38 cDNA. The Flag epitope (-Asp-Tyr-Lys-Asp-Asp-Asp-Aps-Lys-; Immunex Corp.) was inserted between codons 1 and 2 of p38 by insertional overlapping polymerase chain reaction (31). A similar polymerase chain reaction procedure was employed to replace Thr^{180} and Tyr^{182} with Ala and Phe, respectively. The sequence of all plasmids was confirmed by automated sequencing using an Applied Biosystems model 373A machine.

Tissue Culture—COS-1 and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% calf serum (Life

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‖ Investigator of the Howard Hughes Medical Institute. To whom correspondence should be addressed: Howard Hughes Medical Institute, Program in Molecular Medicine, University of Massachusetts Medical School, 373 Plantation St., Worcester, MA 01605.

[1] The abbreviations used are: MAP, mitogen-activated protein; ATF2, activating transcription factor 2; cPLA$_2$, cytoplasmic phospholipase A$_2$; EGF, epidermal growth factor; EGF-R, EGF receptor; GST, glutathione *S*-transferase; JNK, c-Jun NH$_2$-terminal kinase; LPS, lipopolysaccharide; UV, ultraviolet; PAGE, polyacrylamide gel electrophoresis.

Technologies, Inc.). Chinese hamster ovary cells expressing human CD14 (32) were maintained in Ham's F-12 medium supplemented with 5% fetal bovine serum (Life Technologies, Inc.). Transient transfection assays were performed using the lipofectamine reagent according to the manufacturer's recommendations (Life Technologies, Inc.). Phosphate labeling was performed by incubation of cells (4 h) in phosphate-free modified Eagle's medium (Flow Laboratories Inc.) supplemented with 1 mCi/ml [^{32}P]phosphate (DuPont NEN) and 1% fetal bovine serum.

Western Blot Analysis—Proteins were fractionated by SDS-PAGE, electrophoretically transferred to an Immobilon-P membrane, and probed with monoclonal antibodies to phosphotyrosine (PY20) and the Flag epitope (M2). Immunecomplexes were detected using enhanced chemiluminescence (Amersham International PLC).

Immunoprecipitation—The cells were solubilized with lysis buffer (20 mM Tris (pH 7.4), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin) and centrifuged at 15,000 × *g* for 15 min at 4 °C. The epitope-tagged protein kinases were immunoprecipitated by incubation 1 h at 4 °C with the M2 antibody pre-bound to protein-G Sepharose (Pharmacia Biotech Inc.) for 15 min at 22 °C. Endogenous p38 and JNK was immunoprecipitated with polyclonal antibodies pre-bound to protein-A Sepharose (Pharmacia Biotech Inc.) 1 h. The immunoprecipitates were washed twice with lysis buffer.

Binding Assays—Recombinant GST-ATF2 fusion proteins (5 μg) pre-bound to gluthathione-agarose beads were incubated with cell lysates (80 μg) in 500 μl of lysis buffer. After 1 h of incubation at 4 °C, the beads were washed five times with lysis buffer. Protein kinases in the cell lysate and bound to the beads were detected by Western blot analysis.

Protein Phosphorylation—Kinase assays were performed using immunoprecipitates of p38 MAP kinase and JNK. The immunecomplexes were washed twice with kinase buffer (25 mM Hepes (pH 7.4), 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM orthovanadate). The assays were initiated by the addition of 1 μg of substrate protein and 50 μM [γ-^{32}P]ATP (10 Ci/mmol) in a final volume of 25 μl. The reactions were terminated after 30 min at 30 °C by addition of Laemmli sample buffer. The phosphorylation of the substrate proteins was examined after SDS-PAGE by autoradiography and PhosphorImager (Molecular Dynamics Inc.) analysis. Phosphoamino acid analysis was performed by partial acid hydrolysis and thin layer electrophoresis (11).

Immunocytochemistry—Coverslips (22 × 22 mm No. 1; Gold Seal Cover Glass; Becton Dickinson) were pre-treated by boiling in 0.1 N HCl for 10 min, rinsed in distilled water, autoclaved, and coated with 0.01% poly-L-lysine (Sigma). The coverslips were placed at the bottom of 35-mm multiwell tissue culture plates (Becton Dickinson). Transfected COS-1 cells were plated directly on the coverslips and allowed to adhere overnight in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (Life Technologies, Inc.). 24 h post-transfection, the cells were rinsed once and incubated at 37 °C for 30 min in 25 mM Hepes (pH 7.4), 137 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose. The cells were rinsed once with phosphate-buffered saline and the coverslips removed from the tissue culture wells. Cells were fixed in fresh 4% paraformaldehyde in phosphate-buffered saline for 15 min at 22 °C. The cells were permeabilized with 0.25% Triton X-100 in phosphate-buffered saline for 5 min and washed three times in DWB solution (150 mM NaCl, 15 mM sodium citrate (pH 7.0), 2% horse serum, 1% (w/v) bovine serum albumin, 0.05% Triton X-100) for 5 min. The primary antibody (M2 anti-FLAG monoclonal antibody, Eastman-Kodak Co., New Haven, CT) was diluted 1:250 in DWB and applied to the cells in a humidified environment at 22 °C for 1 h. The cells were washed three times and fluorescein isothiocyanate-conjugated goat anti-mouse Ig secondary antibody (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) was applied at a 1:250 dilution for 1 h at 22 °C in a humidified environment. The cells were washed three times in DWB and mounted onto slides with Gel-Mount (Biomeda Corp., Foster City, CA) for immunofluorescence analysis.

Control experiments were performed to assess the specificity of the observed immunofluorescence. No fluorescence was detected when the transfected cells were stained in the absence of the primary M2 monoclonal antibody. In addition, we did not observe fluorescence in experiments using mock-transfected cells. Together, these data demonstrate that the immunofluorescence observed detects the epitope-tagged p38 MAP kinase.

Digital Imaging Microscopy and Image Restoration—Digital images of the fluorescence distribution in single cells were obtained using a Nikon 60x Planapo objective (numerical aperture = 1.4) on a Zeiss IM-35 microscope equipped for epifluorescence as described previously (33, 34). Images of various focal planes were obtained with a computer controlled focus mechanism and a thermoelectrically cooled charged-coupled device camera (model 220; Photometrics Ltd., Tucson, AZ). The exposure of the sample to the excitation source was determined by a computer-controlled shutter and wavelength selector system (MVI, Avon, MA). The charge-coupled device camera and microscope functions were controlled by a microcomputer, and the data acquired from the camera were transferred to a Silicon Graphics model 4D/GTX workstation (Mountainview, CA) for image processing. Images were corrected for non-uniformities in sensitivity and for the dark current of the charge coupled device detector. The callibration of the microscopy blurring was determined by measuring the instrument's point spread function as a series of optical sections at 0.125-μm intervals of a 0.3-μm diameter fluorescently labeled latex bead (Molecular Probes Inc.). The image restoration algorithm used is based upon the theory of ill-posed problems and obtains quantitative dye density values within the cell that are substantially more accurate than those in an unprocessed image (33, 34). After image processing, individual optical sections of cells were inspected and analyzed using computer graphics software on a Silicon Graphics workstation.

RESULTS

Substrate Specificity of p38 MAP Kinase—The p38 MAP kinase shares amino acid sequence similarity with the MAP kinase family of proteins including ERK, JNK, and HOG1 (1). In order to characterize the enzymatic activity of p38, we employed recombinant p38 expressed in *Escherichia coli* to examine the phosphorylation of several proteins that have been demonstrated to be substrates for the ERK and/or JNK groups of MAP kinases. In initial studies we examined the phosphorylation of the ERK substrates myelin basic protein (35) and the EGF-R (36). It was observed that p38 phosphorylated both of these proteins (Fig. 1*A*). In contrast, phosphorylation of the JNK substrate c-Jun (11, 12, 14) was not detected. These data indicate that the substrate specificity of p38 is similar to the ERK group of MAP kinases. However, not all ERK substrates were phosphorylated by p38. For example, the ERK substrates cytoplasmic phospholipase A_2 ($cPLA_2$) (37) and c-Myc (26) were not phosphorylated by p38 (Fig. 1*A*). Together, these data demonstrate that the substrate specificity of p38 differs from both the ERK and JNK groups of MAP kinases.

Although the ERK substrates myelin basic protein and the EGF-R were phosphorylated by p38, it was unclear whether these proteins represent preferred substrates for this protein kinase. We therefore tested several additional proteins as potential substrates for p38. This analysis demonstrated a low level of phosphorylation of IκB (Fig. 1*A*). However, the transcription factor ATF2 was found to be an excellent p38 substrate. Phosphorylation of ATF2 caused by p38 resulted in an electrophoretic mobility shift during polyacrylamide gel electrophoresis. The site(s) of phosphorylation were mapped to the NH_2-terminal activation domain of ATF2 by deletion analysis (data not shown). Interestingly, JNK phosphorylates ATF2 on Thr-69 and Thr-71 (25). We therefore tested the hypothesis that p38 phosphorylates ATF2 on the same sites. It was found that the replacement of Thr-69 and Thr-71 with Ala residues blocked the phosphorylation of ATF2 caused by p38 (Fig. 1*A*). We conclude that p38 phosphorylates ATF2 within the NH_2-terminal activation domain on Thr-69 and Thr-71. Significantly, the phosphorylation of ATF2 on these sites causes increased transcriptional activity (25). Thus, the transcription factor ATF2 is a potential target of signal transduction by p38 MAP kinase and JNK.

It is known that JNK binds to the activation domain of the substrate c-Jun (11, 12, 38–40). By analogy to JNK, it is possible that p38 MAP kinase binds to ATF2. To test this hypothesis, we incubated cell extracts with immobilized GST or GST-ATF2 (activation domain; residues 1–109). The complexes were extensively washed and the bound protein kinases were detected by Western blotting. This analysis demonstrated that

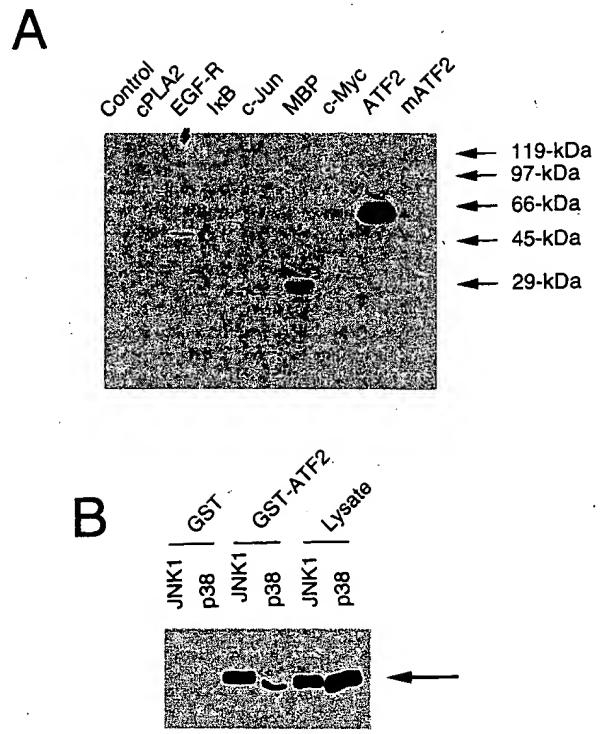

FIG. 1. **Substrate specificity of p38 MAP kinase.** *Panel A*, substrate phosphorylation by p38 MAP kinase was examined by incubation of bacterially-expressed p38 MAP kinase with different proteins and [γ-^{32}P]ATP. The mutated ATF2 protein (*mATF2*) was created by substitution of the phosphorylation sites Thr-69 and Thr-71 with Ala. The phosphorylation reaction was terminated after 30 min by addition of Laemmli sample buffer. The phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography. The rate phosphorylation of the substrate proteins was quantitated by PhosphorImager analysis. The relative phosphorylation of ATF2, myelin basic protein (*MPB*), EGF-R, and IκB was 1.0, 0.23, 0.04, and 0.001, respectively. *Panel B*, cell extracts expressing epitope-tagged JNK1 and p38 MAP kinase were incubated with a GST fusion protein containing the activation domain of ATF2 (residues 1–109) immobilized on gluthathione-agarose. The supernatant was removed and the agarose was washed extensively. Western blot analysis of the supernantant and agarose-bound fractions with the M2 monoclonal antibody was used to detect the protein kinases by enhanced chemiluminescence detection. Control experiments were performed using immobilized GST.

both p38 MAP kinase and JNK bind to the ATF2 activation domain[2] (Fig. 1*B*).

p38 MAP Kinase Is Activated by Pro-inflammatory Cytokines and Environmental Stress—Treatment of cultured cells with EGF or phorbol ester causes maximal activation of the ERK subgroup of MAP kinases (41, 42). However, these treatments cause only a small increase in JNK protein kinase activity (11, 12, 14). Significantly, EGF and phorbol ester caused only a modest increase in p38 protein kinase activity (Figs. 2 and 3). Together, these data indicate that the regulation of p38 may be more similar to JNK than ERK. This hypothesis was confirmed by investigation of the effect of JNK activators on p38 protein kinase activity. It was observed that environmental stress (UV radiation and osmotic shock) caused a marked increase in the activity of both p38 and JNK (Figs. 4 and 5). It was also observed that p38 and JNK were activated in cells treated with pro-inflammatory cytokines (tumor necrosis factor and interleukin-1) or endotoxic LPS (Figs. 6–8). Together, these data indicate that p38 MAP kinase, like JNK, is activated by a stress-induced signal transduction pathway. However, activation of p38 MAP kinase by alternative pathways is not excluded

[2] Control experiments demonstrated that JNK, but not p38 MAP kinase, bound to the activation domain of c-Jun.

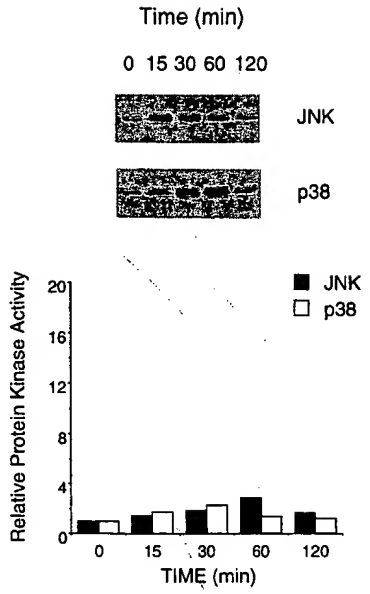

FIG. 2. **Phorbol ester weakly activates p38 MAP kinase.** The activity of p38 MAP kinase and JNK was measured in immunecomplex protein kinase assays using [γ-^{32}P]ATP and ATF2 as substrates. The phosphorylated ATF2 was detected after SDS-PAGE by autoradiography. The figure shows the effect of treatment of HeLa cells with 10 nM phorbol myristate acetate. The rate of phosphorylation was quantitated by PhosphorImager analysis and is presented as the JNK and p38 protein kinase activity relative to control cells treated without agonist (1.0).

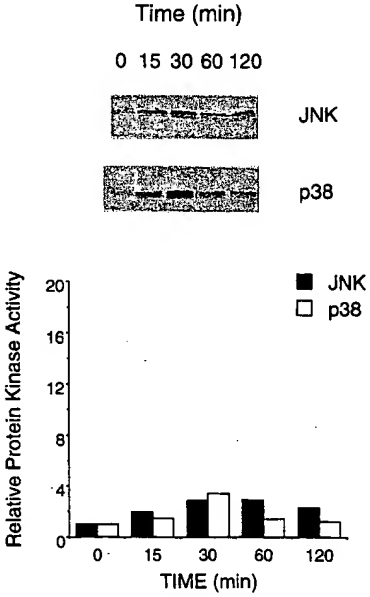

FIG. 3. **EGF weakly activates p38 MAP kinase.** The activity of p38 MAP kinase and JNK was measured in immunecomplex protein kinase assays using [γ-^{32}P]ATP and ATF2 as substrates. The phosphorylated ATF2 was detected after SDS-PAGE by autoradiography. The figure shows the effect of treatment of HeLa cells with 10 nM EGF. The rate of phosphorylation was quantitated by PhosphorImager analysis and is presented as the JNK and p38 protein kinase activity relative to control cells treated without agonist (1.0).

by these data.

p38 MAP Kinase Is Activated by Dual Phosphorylation on Tyr and Thr—ERKs and JNKs are activated by dual phosphorylation within the motifs Thr-Glu-Tyr and Thr-Pro-Tyr, respectively (11, 43). In contrast, the p38 MAP kinase contains the related sequence Thr-Gly-Tyr (19, 20). To test whether this motif is relevant to the activation of p38, we examined the effect of the replacement of Thr-Gly-Tyr with Ala-Gly-Phe. The

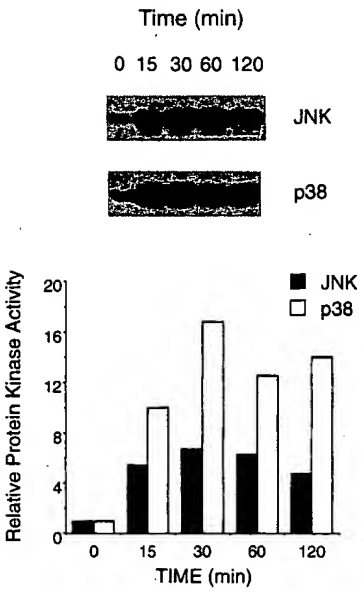

FIG. 4. **UV radiation activates p38 MAP kinase.** The activity of p38 MAP kinase and JNK was measured in immunecomplex protein kinase assays using [γ-^{32}P]ATP and ATF2 as substrates. The phosphorylated ATF2 was detected after SDS-PAGE by autoradiography. The figure shows the effect of treatment of HeLa cells with 40 J/m^2 UV-C. The rate of phosphorylation was quantitated by PhosphorImager analysis and is presented as the JNK and p38 protein kinase activity relative to control cells treated without agonist (1.0).

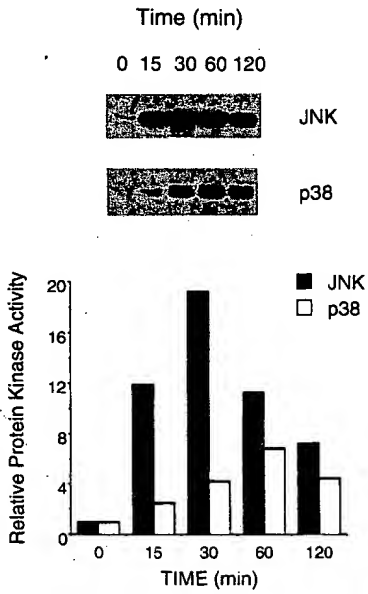

FIG. 5. **Osmotic stress activates p38 MAP kinase.** The activity of p38 MAP kinase and JNK was measured in immunecomplex protein kinase assays using [γ-^{32}P]ATP and ATF2 as substrates. The phosphorylated ATF2 was detected after SDS-PAGE by autoradiography. The figure shows the effect of treatment of HeLa cells with 300 mM sorbitol. The rate of phosphorylation was quantitated by PhosphorImager analysis and is presented as the JNK and p38 protein kinase activity relative to control cells treated without agonist (1.0).

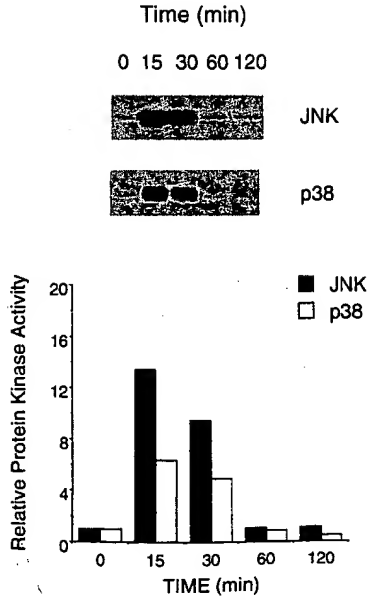

FIG. 6. **Interleukin-1 activates p38 MAP kinase.** The activity of p38 MAP kinase and JNK was measured in immunecomplex protein kinase assays using [γ-^{32}P]ATP and ATF2 as substrates. The phosphorylated ATF2 was detected after SDS-PAGE by autoradiography. The figure shows the effect of treatment of HeLa cells with 10 ng/ml interleukin-1. The rate of phosphorylation was quantitated by PhosphorImager analysis and is presented as the JNK and p38 protein kinase activity relative to control cells treated without agonist (1.0).

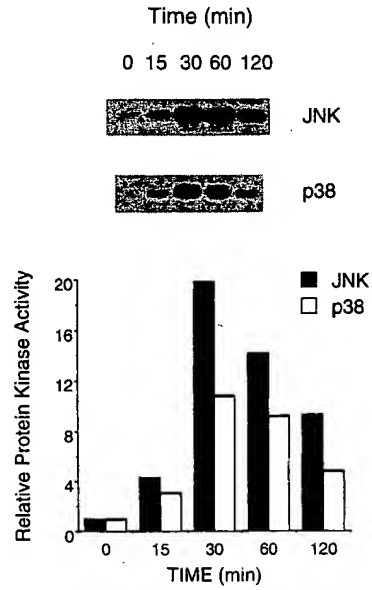

FIG. 7. **Tumor necrosis factor activates p38 MAP kinase.** The activity of p38 MAP kinase and JNK was measured in immunecomplex protein kinase assays using [γ-^{32}P]ATP and ATF2 as substrates. The phosphorylated ATF2 was detected after SDS-PAGE by autoradiography. The figure shows the effect of treatment of HeLa cells with 10 ng/ml tumor necrosis factor α. The rate of phosphorylation was quantitated by PhosphorImager analysis and is presented as the JNK and p38 protein kinase activity relative to control cells treated without agonist (1.0).

wild-type and mutant forms of p38 were expressed at similar levels (Fig. 9*A*). Western blot analysis using an antiphosphotyrosine antibody demonstrated that exposure to UV radiation caused an increase in the Tyr phosphorylation of p38 (Fig. 9*A*). The increased Tyr phosphorylation was confirmed by phosphoamino acid analysis of p38 isolated from [^{32}P]phosphate-labeled cells (Fig. 9*B*). This analysis also demonstrated that UV radiation caused increased Thr phosphorylation of p38 (Fig. 9*B*). Significantly, the increased phosphorylation on Tyr and Thr was blocked by mutation of the dual phosphorylation motif Thr-Gly-Tyr (Fig. 4, *A* and *B*). To examine the signficance of the dual phosphorylation of p38, we measured the protein kinase activity of the wild-type and mutated enzymes. UV radiation caused a marked increase in the activity of wild-type (Thr-Gly-Tyr) p38 (Fig. 9*C*). In contrast, the mutated (Ala-Gly-Phe) p38 was found to be catalytically inactive (Fig. 9*C*). Together, these data demonstrate that p38 is activated by dual phosphoryla-

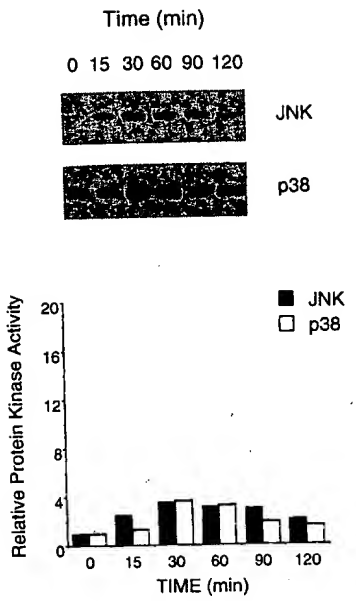

FIG. 8. **LPS activates p38 MAP kinase.** The activity of p38 MAP kinase and JNK1 was examined using Chinese hamster ovary cells that express human CD14. The effect of treatment of the cells with 10 ng/ml LPS is presented. The protein kinase activity was measured in immunecomplex protein kinase assays using [γ-^{32}P]ATP and ATF2 as substrates. The phosphorylated ATF2 was detected after SDS-PAGE by autoradiography. The rate of phosphorylation was quantitated by PhosphorImager analysis and is presented as the JNK and p38 protein kinase activity relative to control cells treated without agonist (1.0).

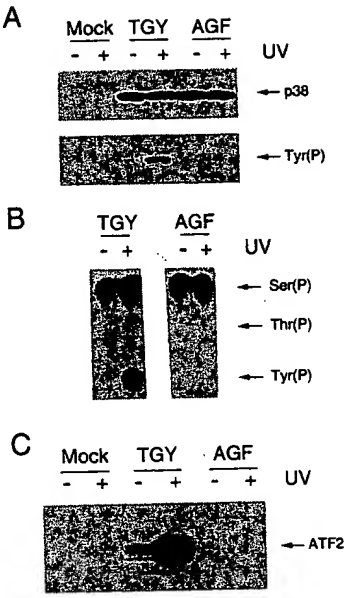

FIG. 9. **Dual phosphorylation on Thr and Tyr is required for p38 MAP kinase activation.** *Panel A*, COS-1 cells expressing wild-type (Thr^{180}-Gly-Tyr^{182}) or mutated (Ala^{180}-Gly-Phe^{182}) p38 MAP kinase were treated without and with UV-C (40 J/m^2). The cells were harvested 30 min following exposure to UV-C radiation. Control experiments were performed using mock-transfected cells. The level of expression of epitope-tagged p38 MAP kinase and the state of Tyr phosphorylation of p38 MAP kinase was examined by Western blot analysis using the M2 monoclonal antibody and the phosphotyrosine monoclonal antibody PY20. Immune complexes were detected by enhanced chemiluminescence. *Panel B*, the p38 MAP kinase was isolated from cells metabolically-labeled with [^{32}P]phosphate by immunoprecipitation with the M2 monoclonal antibody and SDS-PAGE. The p38 MAP kinase phosphorylation was examined by phosphoamino acid analysis. *Panel C*, the p38 MAP kinase was isolated from the COS-1 cells by immunoprecipitation. Protein kinase activity was measured in the immune complex using [γ-^{32}P]ATP and GST-ATF2 as substrates. The phosphorylated GST-ATF2 was detected after SDS-PAGE by autoradiography.

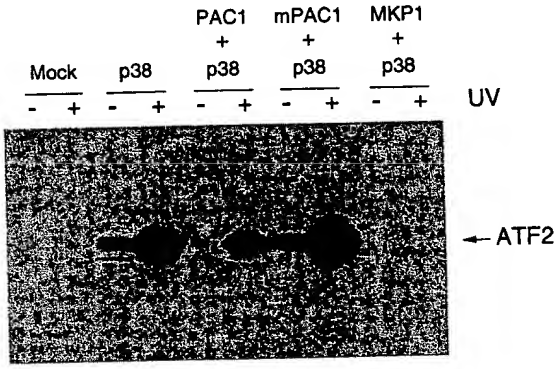

FIG. 10. **MAP kinase phosphatase inhibits p38 MAP kinase activation.** The effect of expression of human MKP1 and PAC1 on p38 MAP kinase activity is presented. The cells were treated without and with 40 J/m^2 UV-C. Control experiments were performed using mock-transfected cells (control) and cells transfected with the catalytically inactive mutated phosphatase mPAC1 (Cys^{257}/Ser). The activity of p38 MAP kinase was measured with an immunecomplex protein kinase assay employing [γ-^{32}P]ATP and GST-ATF2 as substrates.

tion within the motif Thr-Gly-Tyr.

p38 MAP Kinase Is Inhibited by Dual Specificity MAP Kinase Phosphatases—It has recently been demonstrated that ERK activity is regulated by the mitogen-induced dual specificity phosphatases MKP1 and PAC1 (29, 44). The activation of p38 by dual phosphorylation (Fig. 9) suggests that p38 MAP kinase may also be regulated by dual specificity phosphatases. We therefore examined the effect of MKP1 and PAC1 on p38 MAP kinase activation. It was observed that the expression of PAC1 or MKP1 inhibited p38 activity (Fig. 10). The inhibitory effect of MKP1 was greater than PAC1. In contrast, a catalytically inactive mutant phosphatase did not inhibit p38 MAP kinase (Fig. 10). Control experiments demonstrated that these phosphatases did not alter the level of expression of p38 MAP kinase (data not shown). Together, these data demonstrate that p38 MAP kinase can be regulated by the dual specificity phosphatases PAC1 and MKP1.

Subcellular Distribution of p38 MAP Kinase—The subcellular distribution of p38 MAP kinase was examined by indirect immunofluorescence microscopy. Epitope-tagged p38 MAP kinase was detected using the M2 monoclonal antibody. Control experiments demonstrated that no staining of mock-transfected cells was detected. However, specific staining of cells transfected with epitope-tagged p38 MAP kinase was observed (Fig. 11). The p38 MAP kinase was detected at the cell surface, in the cytoplasm, and in the nucleus. Marked changes in cell surface and nuclear p38 MAP kinase were not observed following UV irradiation, but an increase in the localization of cytoplasmic p38 MAP kinase to the perinuclear region was detected (Fig. 11). Together, these data demonstrate that p38 MAP kinase is present in both the nuclear and cytoplasmic compartments of cells and that activation by UV irradiation does not cause marked redistribution of p38 MAP kinase from the cytoplasm to the nucleus.[3] The absence of nuclear redistribution of p38 MAP kinase contrasts with observations reported for the ERK group of MAP kinases (45–48). The ERKs are present in the cytoplasm of quiescent cells and translocate into the nucleus following activation (45–48).

[3] A caveat that must be placed on the interpretation of the immunofluorescence experiments is that the images obtained represent the cellular distribution of over-expressed p38 MAP kinase. The distribution of endogenous p38 MAP kinase may be different.

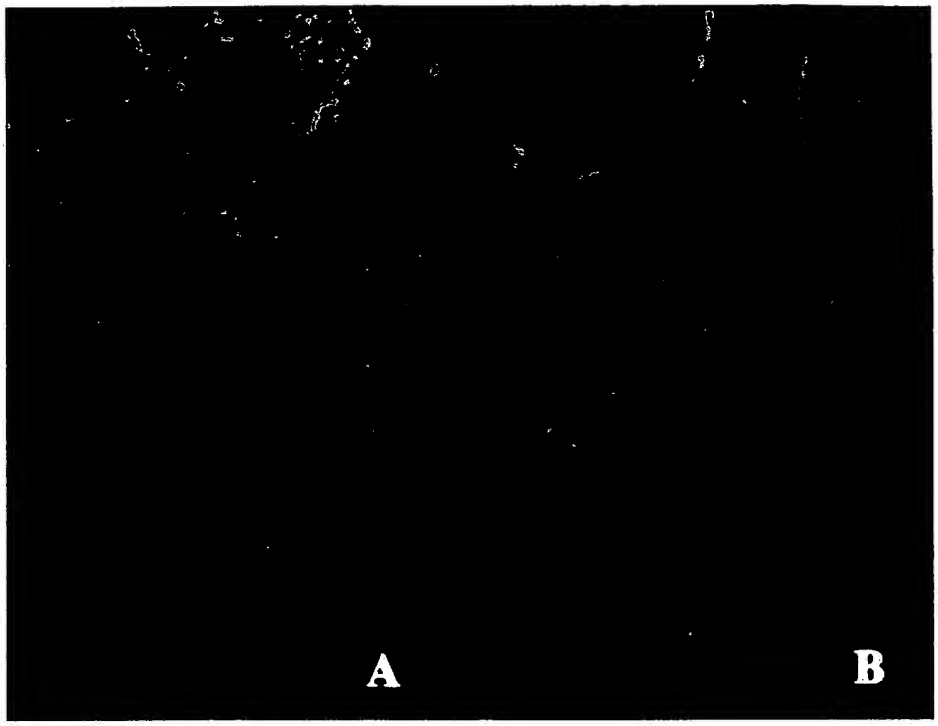

FIG. 11. **Subcellular distribution of p38 MAP kinase.** Epitope-tagged p38 MAP kinase was expressed in COS cells. The cells were treated without (*Panel A*) or with (*Panel B*) 40 J/m^2 UV radiation and then incubated for 60 min at 37 °C. The p38 MAP kinase was detected by indirect immunofluorescence using the M2 monoclonal antibody. The images were acquired by digital imaging microscopy and processed for image restoration.

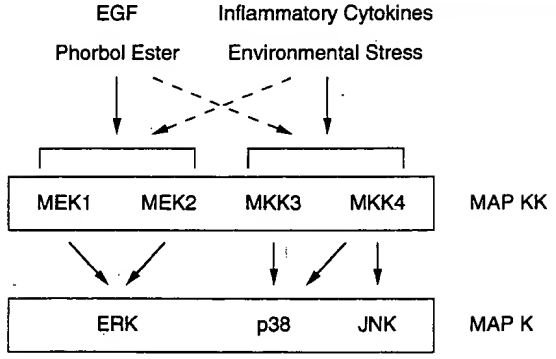

FIG. 12. **Mammalian MAP kinases form an integrated network of signal transduction pathways.** The major stimuli that activate JNK and p38 MAP kinases are pro-inflammatory cytokines and environmental stress. In contrast, the ERK group of MAP kinases are activated in cells treated with EGF or phorbol ester. This difference is accounted for, in part, by the substrate specificities of the MAP kinase kinases MEK1 (53), MEK2 (53), MKK3 (49), and MKK4 (49, 50) which activate ERK, p38, and JNK. These pathways are illustrated schematically.

DISCUSSION

The requirement of dual phosphorylation for activation establishes that p38 is a member of the MAP kinase group of signal transducing proteins (1). However, the absence of detectable phosphorylation of cPLA$_2$, c-Myc, and c-Jun together with the strong phosphorylation of ATF2 indicates that the substrate specificity of p38 differs from both the JNK (11–14, 16, 17, 25) and ERK (41, 42) subgroups of MAP kinase. It is therefore likely that the p38 MAP kinase signal transduction pathway has a distinct function in the cell. Indeed, it has recently been established that p38 may function in a signal transduction pathway that leads to phosphorylation of small heat shock proteins (20, 21) and increased expression of inflammatory cytokines (22).

EGF and phorbol ester are potent activators of the ERK signal transduction pathway (10). However, we found that these treatments did not cause a marked increase in p38 protein kinase activity. These data indicate that the mechanism of activation of p38 is not identical to the ERK group of MAP kinases. In contrast, the pattern of activation of p38 was found to be similar to JNK, a MAP kinase that is potently activated by pro-inflammatory cytokines and environmental stress (1). Thus p38, like JNK, may be regulated, in part, by a stress-activated signal transduction pathway (Figs. 2–8). This conclusion is consistent with the observation that both p38 and JNK1 are able to complement a defect in the expression of the HOG1 stress-activated MAP kinase in yeast (15, 19). Although p38 and JNK both appear to be activated by a stress-induced signal transduction pathway, a significant question remains concerning the organization of these pathways.

The comparison of the regulation of p38 and JNK activation reveals a marked similarity between these protein kinases, but differences in the time course and extent of activation were also observed (Figs. 2–8). These differences indicate that the p38 and JNK pathways may be distinct. Indeed, p38 and JNK could represent parallel stress-activated signal transduction pathways (49). Alternatively, it is possible that p38 and JNK are activated by a common pathway. A rigorous test of these hypotheses requires the molecular cloning of the dual specificity kinase kinases that activate p38 and JNK. Recently, two MAP kinase kinases (MKK3 and MKK4) that activate p38 MAP kinase have been identified (49). MKK3 is specific for p38 MAP kinase (49). In contrast, MKK4 activates both JNK (49, 50) and p38 MAP kinase (49). Thus, p38 and JNK are activated by related MAP kinase kinases (Fig. 12).

The original identification of p38 demonstrated that this protein is tyrosine phosphorylated in LPS-treated cells (19, 51). This study demonstrates that p38 is a member of the MAP kinase group that is activated by dual phosphorylation on Tyr and Thr by a stress-induced signal transduction pathway. Endotoxic LPS, an activator of the p38 MAP kinase pathway, can therefore be considered to be a form of environmental stress that elicits septic shock (52).

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Expression and Activation of Mitogen-Activated Protein Kinase Kinases-3 and -6 in Rheumatoid Arthritis

Martine Chabaud-Riou and Gary S. Firestein

From the Division of Rheumatology, Allergy, and Immunology, University of California at San Diego School of Medicine, La Jolla, California

The p38 mitogen-activated protein (MAP) kinase signal transduction pathway regulates the production of interleukin-1 and tumor necrosis factor-α. p38 kinase inhibitors are effective in animal models of arthritis and are currently being developed in rheumatoid arthritis (RA). However, little is known about the upstream kinases that control the activation of p38 in RA synovium. *In vitro* studies previously identified the MAP kinase kinases (MAPKKs) MKK3 and MKK6 as the primary regulators of p38 phosphorylation and activation. To investigate a potential role for MKK3 and MKK6 in RA, we evaluated their expression and regulation in RA synovium and cultured fibroblast-like synoviocytes (FLS). Immunohistochemistry demonstrated that MKK3 and MKK6 are expressed in RA and osteoarthritis (OA) synovium. Digital image analysis showed no significant differences between OA and RA with regard to expression or distribution. However, phosphorylated MKK3/6 expression was significantly higher in RA synovium and was localized to the sublining mononuclear cells and the intimal lining. Actin-normalized Western blot analysis of synovial tissue lysates confirmed the increased expression of phosphorylated MKK3/6 in RA. Western blot analysis demonstrated constitutive expression of MKK3 and MKK6 in RA and OA FLS. Phospho-MKK3 levels were low in medium-treated FLS, but were rapidly increased by interleukin-1 and tumor necrosis factor-α, although phospho-MKK6 levels only modestly increased. p38 co-immunoprecipitated with MKK3 and MKK6 from cytokine-stimulated FLS and the complex phosphorylated activating transcription factor-2 in an *in vitro* kinase assay. These data are the first documentation of MKK3 and MKK6 activation in human inflammatory disease. By forming a complex with p38 in synovial tissue and FLS, these kinases can potentially be targeted to regulate the production of proinflammatory cytokine production in inflamed synovium. *(Am J Pathol 2004, 164:177–184)*

Mitogen-activated protein (MAP) kinases are a family of serine/threonine kinases that mediate signal transduction and orchestrate an appropriate cellular response to environmental stress. In mammalian cells, three principle MAP kinase pathways have been identified, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38.[1] Multiple MAP kinase pathways can be simultaneously activated and the relative balance is determined by the parallel upstream kinase cascades known as MAP kinase kinases (MAPKKs) and MAP kinase kinase kinases (MAP3Ks).[2]

The p38 MAP kinase is of particular interest in inflammatory diseases such as rheumatoid arthritis (RA) because it regulates the production of pathogenic cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α.[3,4] p38 is expressed and activated in RA synovium[5] and blockade using selective inhibitors decreases inflammation and bone destruction animal models of arthritis.[6] However, little is known about the upstream kinases that can activate this pathway in joint tissues. Of the MAPKKs, MKK3 and MKK6 are thought to be especially important regulators of p38 and represent potential therapeutic targets to modulate cytokine production.[7] MKK6 and MKK3 have significant homology at the amino acid level, with 82% amino acid identity.[8,9] However, there is significantly less nucleotide sequence homology at the DNA level, especially at the C- and N-terminal regions. MKK6 and MKK3 also differ in tissue and cell expression.[10,11] Further diversity is provided by numerous tissue-specific splice variants for MKK6.[12,13]

Both MKK3 and MKK6 are activated upon phosphorylation of serine and threonine residues within subdomain VIII by upstream MAPKK kinases (MAP3Ks).[14] MKK3 selectively phosphorylates p38α, γ, and δ whereas MKK6 activates all four p38 isoforms (α, β, γ, and δ).[15] This suggests that substrate selectivity might contribute to the distinct functional profiles of MKK activation. Additional specificity results from selective activation of different MKKs. For instance, MKK6 is the major activator of p38 in cells exposed to osmotic stress[16] and MKK3 is required

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Address reprint requests to Gary S. Firestein, M.D., Division of Rheumatology, Allergy, and Immunology, UCSD School of Medicine, 9500 Gilman Dr., La Jolla, CA 92093. E-mail: gfirestein@ucsd.edu.

for full activation of p38 MAPK in murine embryonic fibroblasts.[17]

To study the relative contribution of MKK3 and MKK6 in RA, we investigated their expression and function in RA synovial tissue and cultured fibroblast-like synoviocytes (FLS). The data indicate that both MKK3 and MKK6 are activated in RA synovium. However, MKK3 phosphorylation is greater than MKK6 activation in cultured FLS stimulated by IL-1 or TNF-α. Both can form stable signaling complexes with p38 that can phosphorylate downstream substrates. This is the first demonstration of MKK3 and MKK6 activation in human inflammatory disease and suggests that MKK3 or MKK6 are potential therapeutic targets for RA.

Materials and Methods

Cells and Synovial Tissue

FLS were isolated from RA and osteoarthritis (OA) synovial tissues obtained at joint replacement as previously described.[18] The diagnosis of RA conformed to the 1987 revised American College of Rheumatology (ACR) criteria.[19] Briefly, the tissues were minced and incubated with 1 mg/ml of collagenase in serum-free Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Grand Island, NY) for 2 hours at 37°C, filtered through a nylon mesh, extensively washed, and cultured in DMEM supplemented with 10% fetal calf serum (FCS) (endotoxin content <0.006 ng/ml; Life Technologies), penicillin, streptomycin, gentamicin, and L-glutamine in a humidified 5% CO_2 atmosphere. After overnight culture, nonadherent cells were removed and adherent cells were cultivated in DMEM plus 10% FCS. At confluence, cells were trypsinized, split at a 1:3 ratio, and recultured in medium. Synoviocytes were used from passages three through eight in which they comprised a homogeneous population of FLSs (<1% CD11b, <1% phagocytic, and <1% FcgRII receptor-positive).[18] The synovial tissue was snap-frozen and processed for Western blot and immunohistochemistry.

Antibodies and Reagents

Affinity-purified rabbit polyclonal MKK3 antibodies, goat polyclonal MKK6 antibodies, and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal phospho-MKK3/6 and phospho-p38 MAPK antibodies and GST-activating transcription factor (ATF)-2 were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies specific for macrophages (anti-CD68, clone EMBII) and for T cells (anti-CD3, clone UCHTI) were purchased from DAKO (Glostrup, Denmark). Antibody for synovial fibroblasts (anti-CD55, Ab-1) was purchased from Oncogene Research Products (Boston, MA). CD55, which is a decay-activating factor, can be expressed by other cells but has been used routinely to distinguish FLS from macrophages in synovium.[20] IL-1β and TNF-α was purchased from R&D systems (Minneapolis, MN). The p38 inhibitor, SB203580 was purchased from Promega (Madison, WI) and the JNK inhibitor, SP600125, was provided by Celgene, Inc. (San Diego, CA).

Western Blot Analysis

FLS were cultured in DMEM with 10% FCS in 100-mm dishes. At 80% confluency, they were synchronized in DMEM with 0.1% FCS for 48 hours. FLS were then stimulated with IL-1 (2 ng/ml) or TNF-α (10 ng/ml) for up to 24 hours. Cells were then washed twice with phosphate-buffered saline (PBS) and lysed using RIPA buffer [50 mmol/L HEPES, 150 mmol/L NaCl, 1% Triton X-100, 10% glycerol, 1 mmol/L $MgCl_2$, 1.5 mmol/L ethylenediaminetetraacetic acid (pH 8.0), 20 mmol/L β-glycerophosphate, 50 mmol/L NaF, 1 mmol/L Na_3VO_4, 10 μg/ml aprotinin, 1 μmol/L pepstatin A, and 1 mmol/L phenylmethyl sulfonyl fluoride]. For Western blot studies of synovium, the frozen synovial tissue was pulverized and lysed in the same manner. The protein concentrations in the extracts were determined using the DC Protein assay kit (Bio-Rad, Hercules, CA). Whole-cell lysates (50 μg) or tissue lysates (200 μg) were fractionated on Tris-glycine-buffered 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Perkin-Elmer Life Sciences, Inc., Boston, MA). The membranes were blocked with 5% nonfat milk powder for 1 hour at room temperature, followed by incubation with antibody to MKK3, MKK6, phospho-MKK3/6, phospho-MAPK p38, or actin at 4°C overnight. After washing with TBS-T, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. Immunoreactive protein was detected with chemiluminescence and autoradiography (Eastman Kodak Co., Rochester, NY).

Immunoprecipitation and Kinase Assays

To measure the activities of MKK3 and MKK6, FLS were serum-starved (0.1% FCS) for 48 hours and then treated with either low-serum medium or IL-1 for 15 minutes. The cells were then washed three times with ice-cold PBS and lysed in immunoprecipitation buffer (1% Triton X-100, 50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L sodium vanadate, 10 μmol/L leupeptin, and 1.5 μmol/L pepstatin). Lysates were clarified by centrifugation at 15,000 × *g* for 10 minutes. Protein concentration in the supernatant was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc.). The supernatant was precleared with appropriate sera and with protein A- or G-Sepharose (Oncogene Research Products) for 1 hour. Clarified lysates of 500 μg of total protein were incubated with 4 μg of anti-MKK6 or anti-MKK3 antibody for 4 hours, followed by additional incubation with protein A- or G-Sepharose overnight. The immunoprecipitates were washed three times with immunoprecipitation buffer, once with kinase buffer (50 mmol/L HEPES, pH 7.4, 10 mmol/L $MgCl_2$, 0.2 mmol/L dithiothreitol, 1 mmol/L sodium vanadate, 10 μmol/L leupeptin, and 1.5 μmol/L

pepstatin), and resuspended in 25 μl of kinase buffer containing 5 μCi of [γ-32P] ATP, 100 μmol/L ATP, and 4 μg of glutathione S-transferase-ATF-2 (Cell Signaling Technology, Inc., Beverly, MA) and incubated at 37°C for 30 minutes. In some experiments, kinase reactions included p38 (SB203580, 3 μmol/L) or JNK (SP600125, 10 μmol/L) inhibitors. Reactions were stopped by addition of SDS sample buffer (100 mmol/L Tris, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.25% bromophenol blue). After electrophoresis, the gel was subjected to autoradiography. The density of target bands was analyzed using National Institutes of Health Image (version 1.61; National Institutes of Health, Bethesda, MD).

For Western blot analysis, the same protocol was used for immunoprecipitation except that the pellets were washed four times with washing buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L ethylenediaminetetraacetic acid, 1% Triton X-100, 10% glycerol), incubated in 2× nonreducing Laemmli sample buffer, and heated for 5 minutes at 95°C. The samples were processed for SDS-polyacrylamide gel electrophoresis and Western blot analysis.

Immunohistochemistry

Immunohistochemistry was performed as previously described.[21] Cryosections (5 μm) of synovial tissue from RA and OA patients were fixed with acetone or 4% formalin for 10 minutes and then incubated with anti-MKK3, anti-MKK6 or anti-phospho-MKK3/6 antibody overnight at 4°C. Isotype-matched antibodies served as control. Endogenous peroxidase was depleted with 0.1% H_2O_2 and 0.1% NaN_3. The sections were then stained with biotinylated secondary antibody anti-rabbit or anti-goat IgG and Vectastain ABC (Vector, Burlingame, CA) and developed using diaminobenzidine (Vector). The immunostained samples were counterstained with hematoxylin. Antibodies for synovial fibroblasts, macrophages, and T cells were used to characterize the cells expressing phospho-MKK3/6 in double-labeling staining. Alkaline phosphatase-labeled horse anti-mouse IgG (Vector) was applied as second antibody. Color was developed using Blue (Vector) as substrate.

After immunohistochemical staining, quantification of positively stained cells was evaluated on six high-powered fields from each section by computer-assisted image analysis. The images were acquired using a Nikon Eclipse E800 microscope (Nikon Instruments, Inc., Melville, NY) and equipped with a MicroFire digital camera (Olympus, Melville, NY). Digital image acquisition was performed by MicroFire software. Quantitative analysis was performed using ImagePro Plus programs (Media Cybernetics, Inc., Silver Spring, MD). Specific areas of interest were selected, including the total tissue section, the intimal lining, or the sublining. The percentage of region covered by diaminobenzidine was quantified (percent positive area) and the mean optical density of the positive region determined. The latter parameter determines average intensity of the selected color in the region of interest. Relative protein expression was determined by multiplying the percent positive area by the mean optical density.

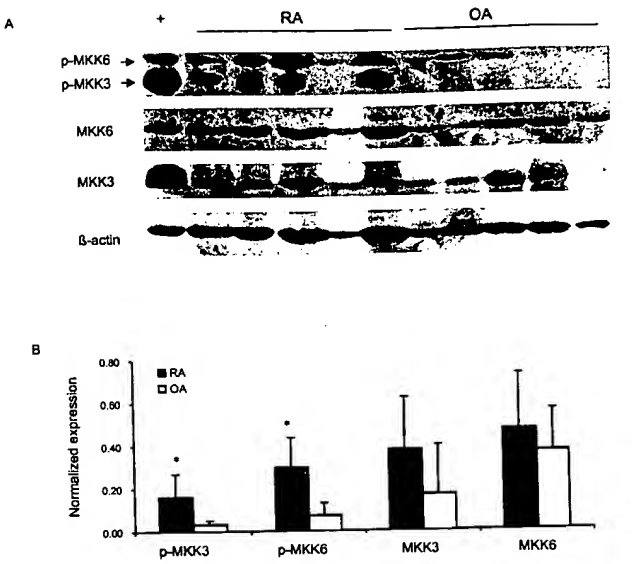

Figure 1. Expression of activated MKK3/6 in synovial tissue from patients with RA and OA. **A:** Western blot analysis was performed with tissue synovial extracts from RA patients ($n = 5$) and OA patients ($n = 5$). Extracts (200 μg) were separated on a 12% SDS-polyacrylamide gel electrophoresis gel, transferred onto polyvinylidene difluoride membranes, and probed with anti-phospho-MKK3/6, MKK3, MKK6, and actin. Phospho-MKK3 and -MKK6 were distinguished on the basis of molecular weight. **B:** The quantitative densitometry analysis of phospho-MKK3/6 expression normalized for actin is shown as the mean ± SD. +, Positive control; *, $P < 0.05$.

Statistical Analysis

Statistics were performed with paired Student's *t*-test. A comparison was considered statistically significant if P was <0.05.

Results

Expression and Activation of MKK3 and MKK6 in Synovial Tissues

To determine whether key upstream activators of p38 are expressed in RA, Western blot studies were initially performed on RA and OA synovial tissue lysates using specific anti-MKK3 and -MKK6 antibodies. The results for synovial extracts from five RA patients and five OA patients are shown in Figure 1A and demonstrate similar levels of expression for both kinases after actin normalization. However, expression of phosphorylated MKK3 and MKK6 was significantly higher in RA than OA synovium ($P < 0.05$). Although no antibodies are available that distinguish between phospho-MKK3 and phospho-MKK6, differences in molecular weight allowed us to evaluate the two kinases separately (molecular weight for MKK3 = 43 kd and MKK6 = 45 kd). Immunohistochemistry studies were then performed to localize MKK3 and MKK6 expression and evaluate relative levels in RA and OA tissues ($n = 6$ each). Both kinases were primarily detected in the synovial intimal lining, although positive

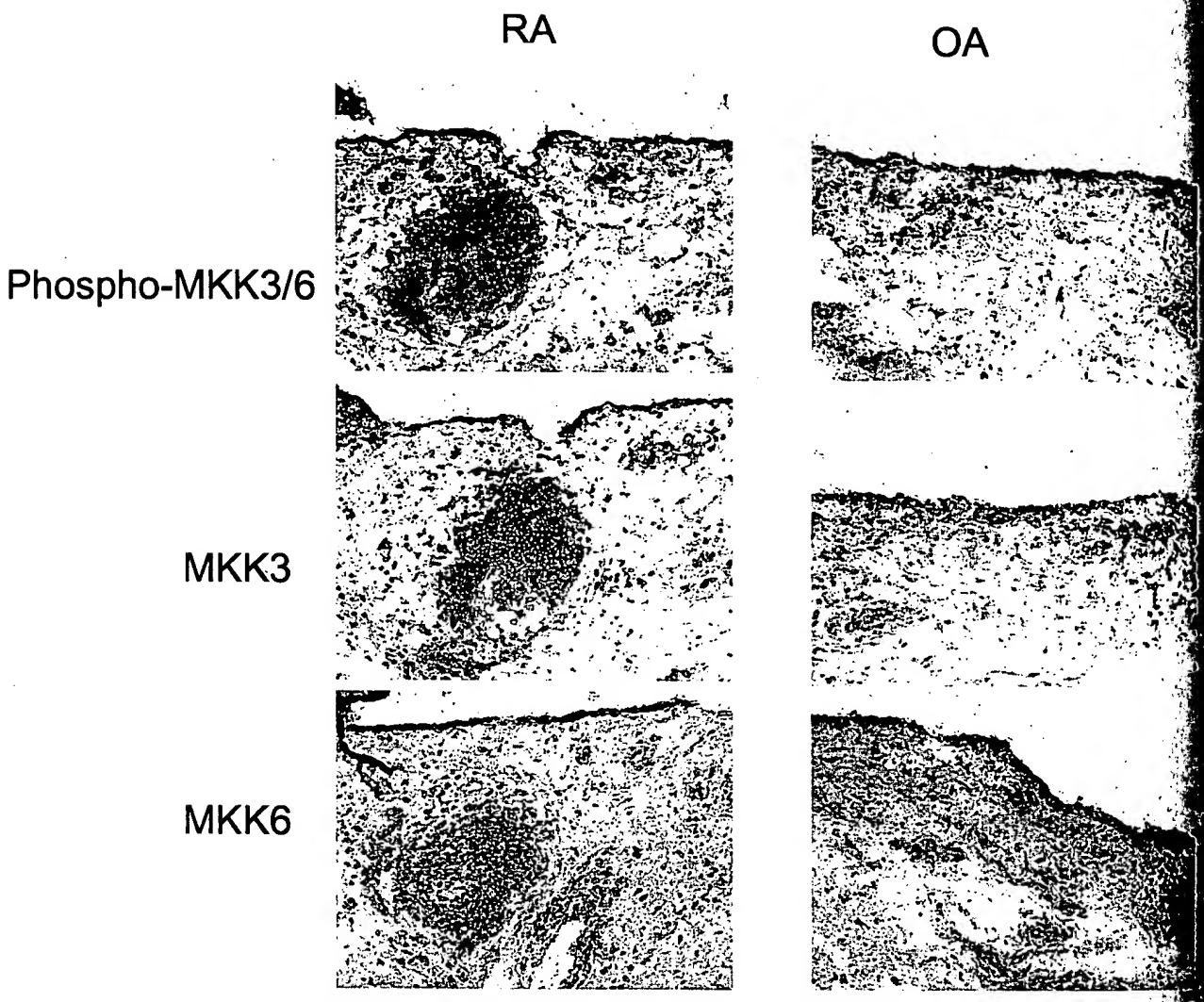

Figure 2. Immunohistochemistry of phospho-MKK3/6 in synovial tissue from RA and OA patients. Phospho-MKK3/6, MKK3, and MKK6 expressions are identified by immunohistochemistry as described in Material and Methods. Staining for MKK3, MKK6, and phospho-MKK3/6 were the highest in the intimal lining. Representative serial sections from a RA and an OA patient are shown. Table 1 shows the results of image analysis. Serial sections stained with control antibodies were negative (data not shown). Original magnifications, ×200.

cells were also identified in the sublining region (see Figure 2 for representative examples). No differences were noted between RA and OA as determined by image analysis (Table 1). Using anti-phospho-MKK3/6 antibodies, however, we observed phospho-MKK3 and -MKK6 expression in the intimal lining and sublining mononu-

Table 1. Digital Image Analysis Results for the Expression of Phospho-MKK3/6, MKK3 and MKK6 in Synovial Tissue

	Lining		Sublining	
	% Stained area	Total expression (pixel units)	% Stained area	Total expression (pixel units)
Phospho-MKK-3/6				
OA (n = 7)	3.4 ± 0.9	383 ± 188	0.6 ± 0.2	45 ± 20
RA (n = 9)	26.2 ± 5.5*	5203 ± 1367*	7.8 ± 1.3†	1952 ± 434†
MKK6				
OA (n = 6)	32.3 ± 5.9	36 ± 9	5.4 ± 1.8	7 ± 2
RA (n = 5)	30.4 ± 7.8	37 ± 9	7.6 ± 1.6	11 ± 3
MKK3				
OA (n = 6)	31.6 ± 5.9	30 ± 7	8.0 ± 2.5	8 ± 3
RA (n = 6)	28.9 ± 5.9	30 ± 7	10.0 ± 1.9	11 ± 2

Percent stained area equals percent of selected region positive for the particular protein.
Total expression equals mean optical density × percent stained area as described in Material and Methods.
Data are shown as mean ± SEM.
*P < 0.01, †P < 0.005 for RA compared with OA.

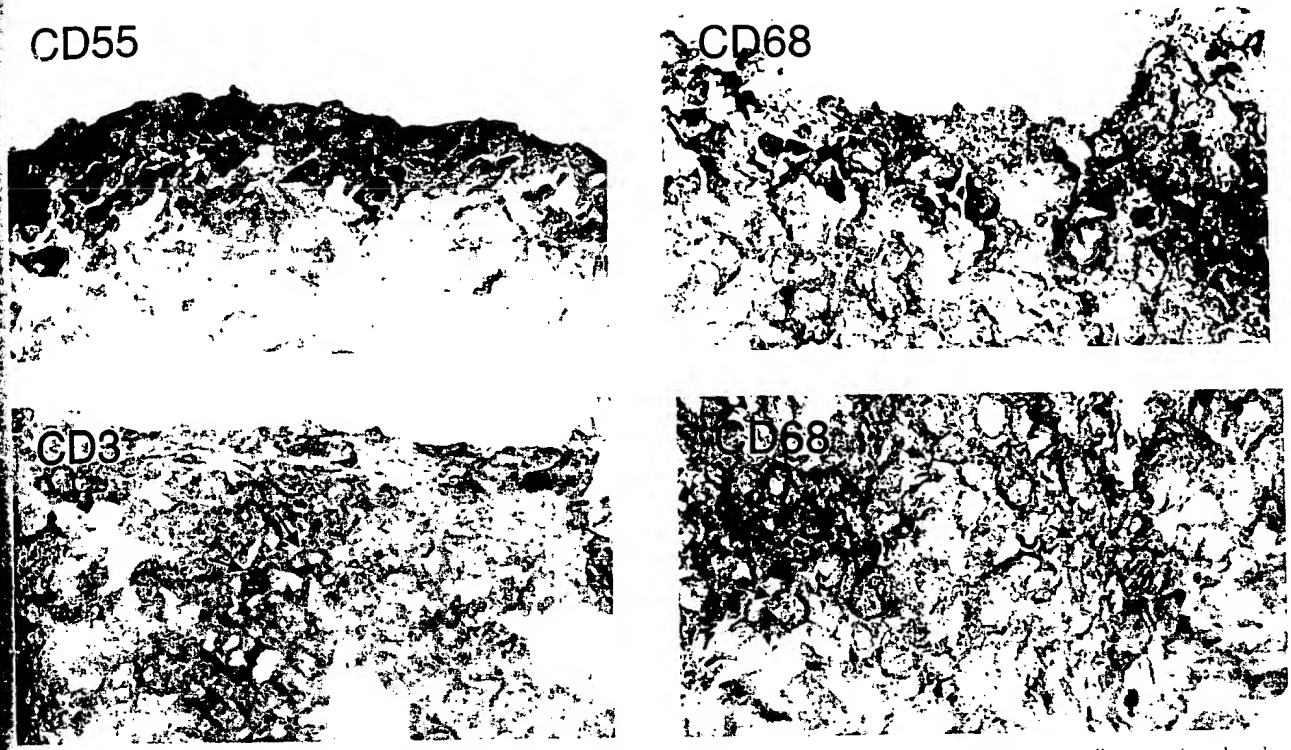

Figure 3. Immunohistochemistry double staining of activated MKK3/6 in synovial tissue from RA synovial tissue. To characterize cells expressing phospho-MKK3/6 (brown), tissue sections were stained with specific antibodies (blue) for fibroblasts (CD55), macrophages (CD68), and T cells (CD3) by double-staining immunohistochemistry as described in Material and Methods. **Arrows** indicate representative double-positive cells. The most intense double staining was observed with intimal lining fibroblasts, followed by macrophages. Rare positive T cells were present in lymphoid aggregates, but T-cell staining was generally weak for the phosphorylated kinases. Serial sections stained with control antibodies were negative (data not shown). Original magnifications, ×40.

clear cells primarily in RA samples. Image analysis demonstrated that both the relative areas of phospho-MKK3/6 and the intensity of staining were significantly higher in RA compared with OA (Table 1). The expression of MKK3, MKK6, and phospho-MKK3/6 is higher in lining compared with the sublining ($P < 0.05$). Double-staining experiments with CD55 and CD68 showed activated MKK3/6 expressed predominantly in synovial fibroblasts in the lining (see Figure 3 for representative examples). In sublining, macrophages expressed predominantly phospho-MKK3/6. Rare sublining T cells contained activated MKK3/6 (Figure 3), although most did not stain with the anti-phospho-MKK3/6 antibody.

Expression and Activation of MKK3 and MKK6 in FLS

Because MKK3 and MKK6 activation was highest in the synovial intimal lining, we subsequently examined the expression of these cytokines in cultured synoviocytes. As anticipated, Western blot demonstrated that MKK3 and MKK6 are constitutively expressed in both RA and OA FLS ($n = 3$ each). The ability of IL-1 and TNF-α to induce phosphorylation of each of these kinases was investigated in a time-course study. FLS were stimulated with IL-1 (2 ng/ml) or TNF-α (10 ng/ml) for up to 24 hours and phosphorylation of MKK3/MKK6 was determined by Western blot analysis. As shown in Figure 4, modest MKK3 activation was detected under resting conditions and was significantly augmented by IL-1 and TNF-α treatment. A significant increase in MKK3 phosphorylation was detected within 15 minutes, with a 25-fold increase with IL-1 and a ninefold increase with TNF-α ($P < 0.001$ compared with medium alone). Of interest, MKK6 phosphorylation was low or undetectable in resting cells and a very modest increase was detected after cytokine stimulation (2.5-fold increase, $P < 0.05$). Note that the time course for activation for MKK3 and MKK6 is similar to p38. These data suggest that phosphorylation of MKK3

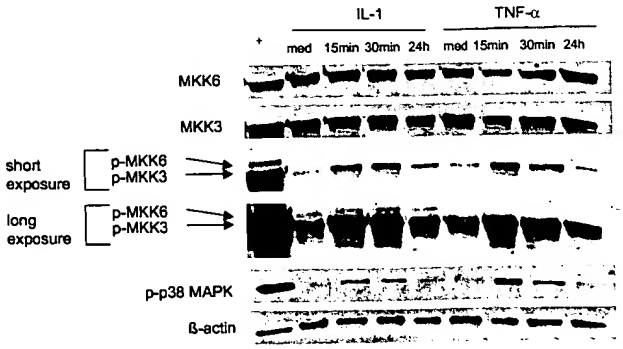

Figure 4. Time course of IL-1 and TNF-α treatment on the protein expression of MKK3, MKK6, and phospho-MKK3/6 in FLS. Cultured FLS were stimulated with IL-1 (2 ng/ml) or TNF-α (10 ng/ml) for the indicated times. The levels of MKK3, MKK6, phospho-MKK3/6, and phospho-p38 (MAPK) were analyzed by Western blot analysis. UV-treated NIH3T3 is a positive control (+) for activation of MKK3 and MKK6. Note that an increase in phospho-MKK3 occurs within 15 minutes while minimal phospho-MKK6 is detected. A longer exposure is shown to demonstrate the faint phospho-MKK6 band. For comparison, phospho-p38 expression is shown in the same experiment. No change is observed in total MKK3 or MKK6 levels. This is representative of three independent experiments with similar results. Med, Medium.

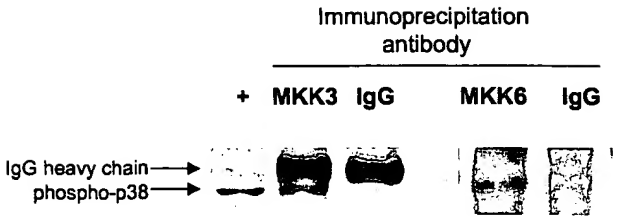

Figure 5. MKK3 and MKK6 form stable complexes with p38 in FLS. RA FLS were stimulated with medium or 2 ng/ml of IL-1 for 15 minutes. Total protein was extracted and immunoprecipitated with antibodies to MKK3 or MKK6 or an IgG control. Western blot analysis was performed on immunoprecipitates to detect p38 MAPK. Representative example is shown. Note that IgG heavy chain is observed in the MKK3 immunoprecipitate and a faint 35- to 40-kd band is observed in the IgG immunoprecipitate in the MKK6 experiment. This is representative of two independent experiments with similar results.

rather than MKK6 is the preferred activation pathway in FLS after cytokine stimulation. There were no significant differences observed between OA and RA FLS with regard of the timing or extent of phosphorylation (data not shown).

MKK3 and MKK6 Form Stable Complexes with p38

Because MKK3 and MKK6 have the capacity to activate p38 MAPK, we then determined if stable complexes between the kinases form in cultured FLS. Previous studies in FLS have demonstrated other complexes between MAPKKs and MAPKs, such as MKK4, MKK7, and JNK.[22] Immunoprecipitation studies were performed using antibodies to either MKK3 or MKK6, followed by Western blot analysis to determine whether phospho-p38 is present in the complexes. As shown in Figure 5, phospho-p38 was readily detected in MKK3 and MKK6 immunoprecipitates but not in precipitates using control antibodies.

MKK3 and MKK6 Function in Activated FLS

To evaluate kinase function, resting and IL-1-stimulated synoviocytes were lysed and MKK3 was recovered by immunoprecipitation. The immunoprecipitated MKK3 was incubated in the presence of the p38 substrate ATF-2 and ^{32}P-ATP and resolved by SDS-polyacrylamide gel electrophoresis. Although ATF-2 is also a substrate for JNK, it is not directly phosphorylated by MKK3 or MKK6. Therefore, any kinase activity in the immunoprecipitates would require the presence of activated MAP kinase. As shown in Figure 6, IL-1 increased ATF-2 phosphorylation approximately fourfold in the MKK3 complexes compared with the control cells ($P < 0.05$). To confirm that p38 in the complex is responsible, selective inhibitors of JNK (SP600125) or p38 (SB203580) were added to the kinase reaction in some experiments. Only the SB203580 significantly decreased the ATF-2-phosphorylating activity indicating that p38 is responsible (2.5-fold decrease, $P < 0.05$). Similar experiments were performed with immunoprecipitated MKK6 (Figure 6, C and D). Although phosphorylated MKK6 was not noted by Western blot (see above), the more sensitive kinase assay was able to detect MKK6-mediated kinase activity. IL-1 also significantly increased ATF-2 phosphorylation,

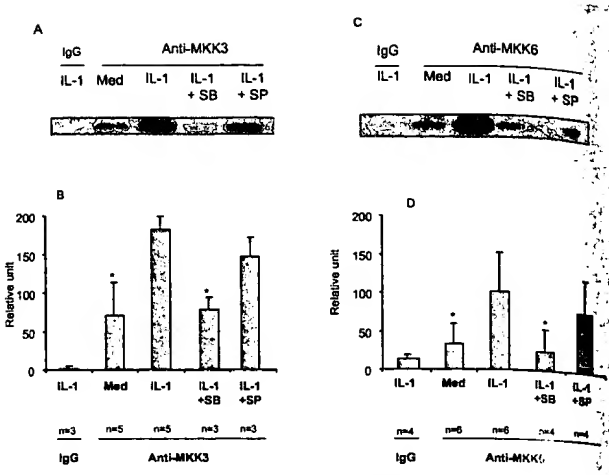

Figure 6. *In vitro* activation of MKK3 and MKK6. FLS were treated with IL-1 (2 ng/ml) and MKK3 and MKK6 activity was measured by immune complex kinase assay using ATF-2 protein as a substrate. The cell lysates were immunoprecipitated with anti-MKK3 or anti-MKK6 antibody or control IgG. **A** and **C:** Kinase assays demonstrated induction of ATF-2-phosphorylating activity in the MKK3 and MKK6 immunoprecipitates. Note that a faint band is observed in the IgG control lane for the MKK6 experiment. **B** and **D:** The quantitative densitometry data are presented as mean ± SD. The p38 inhibitor SB203580 completely blocked kinase activity of the immunoprecipitate. Although modest inhibition of phosphorylating activity was observed in some experiments using the JNK inhibitor SP600125 (MKK6 in **C**), overall the differences were not statistically significant. *, $P < 0.05$. SB, SB203580 (3 μmol/L); SP, SP600125 (10 μmol/L); Med, medium.

which was blocked by the p38 inhibitor (ninefold decrease, $P < 0.05$). Modest inhibition of phosphorylating activity was occasionally observed with the JNK inhibitor SP600125 (MKK6 in Figure 6C), although the differences were not statistically significant. Therefore, both MKK3 and MKK6 form stable complexes with p38 in FLS that can phosphorylate ATF-2.

Discussion

RA is a chronic inflammatory disease marked by synovial hyperplasia with local invasion of bone and cartilage. This disorder is regulated by proinflammatory cytokines such as IL-1 and TNF-α that can activate a broad array of intracellular signal transduction mechanisms.[23–25] Of the cytokine- and stress-activated pathways, MAP kinases are especially important in synoviocytes and chondrocytes because they can increase production of several mediators of inflammation and cartilage damage.[26] The MAP kinase families phosphorylate a number of transcription factors such as activator protein-1 (AP-1), ATF-1 and ATF-2, with subsequent activation of matrix metalloproteinase and cytokine gene expression.[27] p38 MAPK, in particular, can regulate cytokine production through a variety of transcriptional and translational mechanisms.[28,29] Furthermore, p38 participates in other inflammation-related events, such as neutrophil activation,[4] apoptosis,[12] and nitric oxide synthase induction.[30] Inhibition of p38 MAPK with the commonly used SB203580 reduces proinflammatory cytokine production in monocytes/macrophages, neutrophils, and T lymphocytes.[31] In a rodent model of RA, p38 inhibition suppresses inflammation and bone destruction.[6]

p38 MAPK has two main upstream activators: MKK3 and MKK6.[7,8,32,33] Studies in MKK3 knockout and MKK6 knockout mice demonstrate that both are essential for full p38 MAPK activation *in vivo*.[34] MKK4 also phosphorylates p38 *in vitro* when overexpressed in mammalian cells.[32,35] However, the role of MKK4 as an activator of p38 MAPK *in vivo* is unclear. p38 is activated in the rheumatoid synovium,[5,36] although there is little information on the upstream kinases that contribute. The present study was designed to evaluate the potential role of two main upstream activators, MKK3 and MKK6 in RA. Western blot analysis demonstrated that both MKK3 and MKK6 are expressed in OA and RA synovial tissue, with no differences observed between OA and RA. This result was confirmed by quantitative immunohistochemistry, which identified MKK3 and MKK6 in the intimal lining and sublining region of synovial tissue. In contrast, phospho-MKK3/6 expression was markedly greater in RA than in OA synovium, especially in the intimal lining. High levels of activated MKKs were confirmed by Western blot analysis, with increases in both phospho-MKK3 and -MKK6. Because p38 activation contributes to bone destruction and synovial inflammation in animal models of arthritis,[37] one can infer that similar pathways might be relevant in RA. If so, MKK3 and MKK6 are potential gateways to p38 activation in rheumatoid synovium and could enhance cytokine and protease production.

Double-staining experiments with specific markers demonstrated the expression of activated MKK3/6 expression predominantly in synovial fibroblasts in the RA synovial intimal lining where the destructive process is mediated. The regulation of MKK3 and MKK6 was therefore examined in cultured FLS. OA and RA FLS constitutively express MKK3 and MKK6, with no differences observed between cells derived from patients with the two diseases. Activation of MKK3 was rapidly increased by IL-1 or TNF-α, as determined by Western blot analysis and an anti-phospho-MKK antibody. In contrast to intact synovium where similar levels of phospho-MKK3 and -MKK6 were observed, cytokine stimulation only modestly increased MKK6 phosphorylation. These data suggest that MKK3 is the dominant p38-related MAPKK that is activated after cytokine stimulation. The observation is consistent with the fact that *Mkk3*, but not *Mkk6*, gene disruption reduces activation of p38 MAPK and inflammatory cytokine expression in TNF-α stimulated murine fibroblasts.[17] Because phospho-MKK3 and -MKK6 levels are actually similar in RA synovium, the latter is probably expressed in macrophages and other cells in the tissue.

The role of the different MAPKKs depends on the specific MKK activated and may be cell type-specific. Although MKK6 activates all four family members of p38 (α, β, γ, and δ), MKK3 selectively phosphorylates p38α, γ, and δ.[15] MKK6 is the principal activator of p38δ in epithelial cells,[38] whereas MKK3 appears to be an important activator of p38α in murine peritoneal macrophages.[10] In contrast, both the p38α and δ isoforms are activated by MKK3 in murine mesangial cells stimulated by TGF-β1.[39] The relative expression of p38 isoforms in RA has not been fully explored, although p38α and δ are especially prevalent at sites of joint destruction.[5,36]

To facilitate signal transduction, the enzymes can be organized into functional units that include both upstream and downstream members of the family along with various scaffold proteins.[40,41] For instance, we have recently described an activated tri-molecular complex in RA FLS that includes JNK and two MAPKKs (MKK4 and MKK7).[22] Our immunoprecipitation experiments also demonstrate MKK3-p38 and MKK6-p38 complexes in synoviocytes as observed in some other cell lineages.[41–43] The function of MKK3 and MKK6 in FLS was evaluated by determining the ability of the MKK-p38 complexes to phosphorylate ATF-2 in kinase assays. These studies clearly showed that the complexes are functionally active and that cytokine stimulation leads to activation of both the MKK and p38. Surprisingly, the MKK6 immunoprecipitate from cytokine-activated FLS also phosphorylated ATF-2 even though minimal amounts of phospho-MKK6 were detected. This is probably because of the much greater sensitivity of kinase assays compared with Western blots. The kinase activity of both complexes was blocked *in vitro* by the p38 inhibitor SB203580, indicating that the ATF-2 phosphorylating activity was mediated by p38. The JNK inhibitor modestly decreased kinase function in some experiments, although this was not statistically significant.

In conclusion, these studies demonstrate that MKK3 and MKK6 are important regulators of p38 in FLS and are activated in the synovium of patients. Because this pathway may be a critical regulator of joint destruction and inflammation, MAPKKs are potential therapeutic targets. MKK3 appears to be highly activated in cytokine-activated FLS and might be an especially attractive target for RA. Because alternative pathways for p38 activation are available in other cell types, MKK3 or MKK6 inhibitors could have distinct safety and efficacy profiles compared with a selective p38 inhibitor.

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